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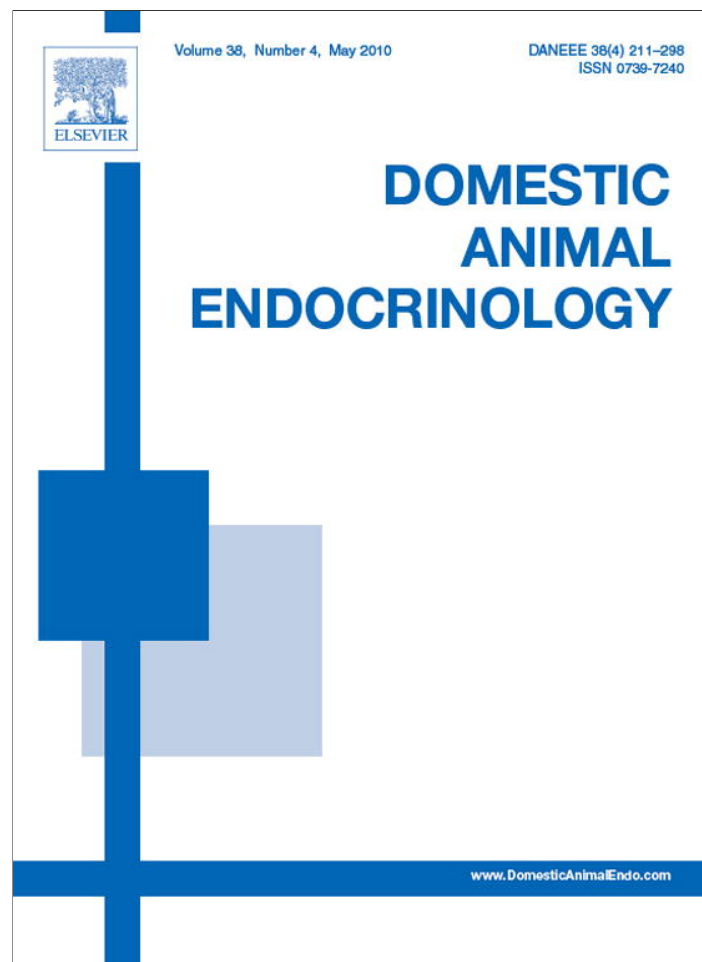
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Platelet-derived growth factor acts via both the Rho-kinase and p38 signaling enzymes to stimulate contraction in an *in vitro* model of equine wound healing

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Abstract

Horses are more prone to complications in the wound healing process than other species, and problems such as chronic inflammation, delayed epithelialization, poor wound contraction, and exuberant granulation tissue are commonly seen, particularly in wounds on the distal limbs. In comparison, wounds of the oral mucosa heal rapidly in a scarless fashion with a high degree of wound contraction. The effect of platelet-derived growth factor BB (PDGF), insulin-like growth factor (IGF)-1, and transforming growth factor β 1 (TGF β 1) on the contraction of a fibroblast-populated collagen matrix (FPCM) as a model of equine wound contraction was investigated using equine oral fibroblasts. The fibroblasts were embedded into floating FPCM and treated with PDGF, IGF-1, and TGF β 1. The surface areas of the FPCM were determined daily for 5 d. Platelet-derived growth factor significantly stimulated the contraction of the FPCM at an optimal concentration of 10 ng/mL ($P=0.025$). Insulin-like growth factor-1 and TGF β 1 did not significantly affect the contraction of the FPCM relative to the control. To elucidate the mechanisms by which PDGF stimulated contraction of FPCM, the Rho-kinase and p38 cell signaling pathways were blocked, resulting in a significant inhibition ($P<0.001$) of PDGF-stimulated contraction. Platelet-derived growth factor BB is a potent stimulator of fibroblast migration, and hence the FPCM contraction generated here is probably a result of its effects on cell migration. The results of the present experiment suggest that this effect is stimulated via both the Rho-kinase and p38 signaling pathways in equine oral fibroblasts.

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Keywords: equine fibroblasts; wound contraction; platelet-derived growth factor; insulin-like growth factor 1; transforming growth factor β 1; Rho-kinase

1. Introduction

Wound healing in horses is more problematic than in other species because of the common problems of chronic inflammation, exuberant granulation tissue, and poor wound contraction [1]. Distal limb wounds are particularly prone to slow healing with unsightly scar

formation, but in comparison, oral wounds heal in a scarless fashion [2,3]. Wound contraction is an important process in determining the strength and size of the final scar tissue, as it pulls the uninjured tissue from the peri-wound area to cover the wound [4]. Therefore, manipulation of wound contraction offers an attractive method for increasing the speed and quality of healing.

There are two main processes that work together to bring about wound contraction: contraction resulting from generation of tractional force by migrating fibroblasts or contraction as a result of myofibroblast contraction [5]. Wound contraction is initiated by fibrob-

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lasts producing tractional force as they migrate into the provisional matrix of the wound around day 5 post-injury under the influence of a variety of growth factors, including platelet-derived growth factor BB (PDGF) and insulin-like growth factor (IGF) [6]. This migration is controlled by various cell signaling pathways including Rho-kinase and p38 [7–9]. The initiation of contraction via the tractional force generated by fibroblasts migrating through the provisional extracellular matrix (ECM) increases tension within the wound to the threshold required to trigger fibroblasts to differentiate into myofibroblasts [10]. Although myofibroblast differentiation depends on a number of factors, including the presence of transforming growth factor β 1 (TGF β 1) and the ED-A splice variant of fibronectin, neither of these alone is sufficient to trigger differentiation in the absence of matrix tension of approximately 20,000 Pa [11–13]. Thus the increase in tension as a result of tractional force is an essential aspect of wound contraction, and therefore the generation of tractional force as a result of the application of different growth factors was the primary focus of this research.

Although a large number of hormones and growth factors are known to affect wound healing, there is currently no published research examining the effect of PDGF, IGF-1, or TGF β 1 on *in vitro* models of wound contraction in horses, nor is there any research examining the ability of equine oral mucosal cells to contract fibroblast-populated collagen matrices (FPCM). Therefore, the objective of the first experiment was to determine the effect of these growth factors on the contraction of floating FPCM containing equine cells harvested from the mucosal surface of the buccal cavity, as understanding the mechanisms that lead to scarless healing may provide insight into methods to decrease scarring in other sites of the body, particularly the limbs. Floating FPCM contract as a result of tractional force generated by migrating fibroblasts rearranging the collagen fibers into a more orderly pattern, and the fibroblasts do not differentiate into myofibroblasts [14]. Whilst *in vivo*, fibroblasts are stimulated to migrate in a directional manner under the influence of growth factors and ECM proteins [6], and the migration within the FPCM is in a nondirectional manner, the mechanisms by which the tractional force is generated and the collagen rearranged are the same, thus making FPCM a suitable model to investigate the role of tractional force in the initiation of wound contraction. Based on the results of the first experiment, the objective of the second experiment was to inhibit the intracellular signaling enzymes Rho-kinase and p38 to determine their importance in PDGF-stimulated contraction of FPCM.

2. Materials and methods

All chemicals were purchased from Sigma Aldrich (Poole, UK).

2.1. Initial isolation and establishment of clonal equine oral mucosal fibroblasts

Fibroblast cultures were established from the mucosal surface of the buccal cavity of 4 horses immediately following euthanasia for unrelated reasons. There were no obvious signs of wounds, scars, or dermatological conditions on the tissue harvested. Samples were transported to the laboratory on ice in tubes containing serum-free Dulbecco's modified Eagle's medium (DMEM) with 5% penicillin/streptomycin (P/S) and 5% amphotericin B. Within 3 h, the skin was washed in phosphate-buffered saline (PBS) containing 5% P/S and 5% amphotericin B. Subcutaneous tissue was removed and the samples minced into approximately 1–2 mm² pieces. Samples were incubated for 45 min in 0.25% trypsin at 37 °C in a shaking incubator. The cell suspension was filtered through a steel mesh before centrifugation at 1,000 rpm for 5 min at 4 °C. The pellet was resuspended in DMEM and centrifuged 2 more times. Cells were plated onto tissue culture flasks and grown in DMEM with 10% fetal bovine serum (FBS) and 5% P/S and 5% amphotericin B at 37 °C in 5% CO₂. Antibiotics were removed from the medium after the first week. Medium was changed at 48–72-h intervals, and cells were passaged at confluency by treatment with 0.05% trypsin/EDTA for 5 min. Unless otherwise stated, throughout the study, cells were plated in flasks at a concentration of 1.0×10^4 cells/cm², and cell numbers were determined using a hemocytometer.

This primary culture was then cloned to increase genetic stability through a process of limiting dilution. This process involved detaching adherent cells with 0.05% trypsin/EDTA for 5 min at the second passage. Trypsin was subsequently inhibited by the addition of DMEM containing 10% FBS, and the cells were gently, but thoroughly, pipetted to ensure dissociation of clumps of cells. Cells were then diluted to 10 cells/mL in DMEM plus 10% FBS via multiple dilutions. A volume of 200 μ L was then added to each well of a 96-well plate. Wells containing a single colony grown from a single cell were first multiplied up into a 24-well plate, then a 6-well plate, before growing in a 25 cm² flask. When confluent, excess cells were cryopreserved in 10% dimethyl sulphoxide (DMSO)/dextran solution (containing 10 g dextran/50 mL DMSO) and 90% FBS. One clone (based on proliferative activity and stability of karyotype number) was selected for future experiments.

2.2. Experiment 1: Effect of growth factors on FPCM contraction

Floating FPCM were prepared by mixing 0.762 mL of $2 \times$ concentrated DMEM, with 4.02 mL of collagen dissolved in 20 mmol acetic acid (2 mg collagen/mL) neutralized with 0.2 mL NaOH, to which 3.96×10^5 oral fibroblasts were added. The final concentration of fibroblasts was 8.3×10^4 cells/mL and 1.6 mg/mL collagen. A volume of 0.4 mL of this collagen and cell suspension was then pipetted into 12 wells of a 48-well plate. Matrices were allowed to set in an incubator for 30 min at 37°C and 5% CO_2 . Once set, each matrix was carefully transferred to a well of a 24-well plate. A volume of 1 mL DMEM containing 2% FBS and the appropriate treatment was added to each well. The treatments were: no addition (control); PDGF-BB (10, 50, or 100 ng/mL); IGF-1 (20, 100, or 400 ng/mL); or TGF β 1 (2, 10, or 20 ng/mL). All growth factors were human in origin. Growth factor concentrations were chosen based on the concentrations most commonly used in previously published literature [15–17]. To make the imaging of the gels easier, phenol red-free DMEM was used throughout. The matrices were scanned daily for 5 d using a BioRad GS-800 calibrated densitometer, and the surface area was determined using Quantity One software (Build 4.5.0, Bio-Rad Laboratories, Watford, UK, 2003).

2.3. Experiment 2: Effect of inhibiting Rho-kinase and p38 on PDGF-stimulated FPCM contraction

The effect of inhibiting Rho-kinase and p38 signaling enzymes on the contraction of PDGF-stimulated floating FPCM was investigated by inclusion of signaling inhibitors in the FPCM medium. Two different inhibitors were used for each pathway to account for different levels of inhibition specificity [18]. The preparation of the FPCM was slightly modified from that of Experiment 1 to create larger FPCM. For each plate, 9.0×10^5 fibroblasts, 1.715 mL of $2 \times$ concentrated DMEM, 9.06 mL of collagen, and 0.45 mL NaOH were mixed. A volume of 0.6 mL of the collagen/cell solution was pipetted into 18 wells of a 24-well plate and when set, the solution was transferred to a 12-well plate. The final concentrations of collagen and cells in the FPCM were the same as for Experiment 1.

For each signaling pathway investigated, 3 controls were set up: 2% FBS only, and 2% FBS plus one of the two inhibitors for the pathway being investigated. For Rho-kinase, the inhibitors used were Y-27632 (10 μM) and hydroxyfasudil (20 μM), and for p38, SB203580 (10 μM) and SB202190 (10 μM) in 0.1% DMSO were

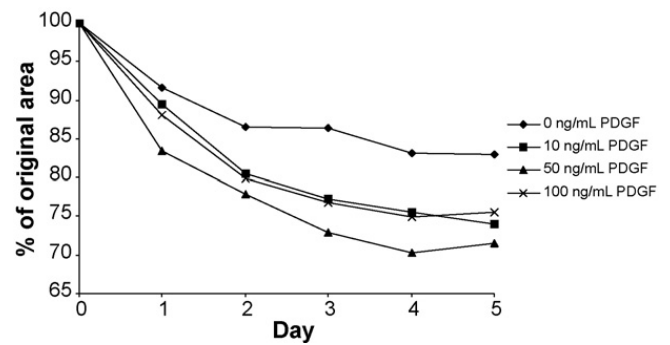


Fig. 1. Effect of platelet-derived growth factor BB on the relative surface area of fibroblast-populated collagen matrix. Least significant difference: 7.313. Platelet-derived growth factor BB (10 ng/mL) caused a significantly greater ($P = 0.025$) contraction compared to the control (0 ng/mL); there was no significant difference between the 10, 50, and 100 ng/mL treatments.

used. Additionally, a positive control of 10 ng/mL PDGF with 2% FBS was used in each case. Finally, for each pathway, FPCM were set up containing 10 ng/mL PDGF with 2% FBS plus one of the two inhibitors. All inhibitors were well characterized for their specificity of inhibition [18].

2.4. Calculations and statistical analysis

The surface areas of the matrices were expressed as a percentage of the original area on day 0, and statistical analyses were carried out using these percentages. All experiments were replicated 3 times from a separate batch of cells, with each replicate containing each treatment in triplicate. Results for each individual growth factor or enzyme were analyzed using split-plot analysis of variance; the main effect was treatment and the split plot was day of incubation. Differences of $P \leq 0.05$ were taken as significant. Significant differences were determined using least significant difference (LSD), with a percentage difference between two results greater than the LSD taken as significant.

3. Results

3.1. Experiment 1: Effect of growth factors on FPCM contraction

Platelet-derived growth factor stimulated the contraction of the FPCM relative to the control at all concentrations investigated ($P = 0.025$; Fig. 1). However, there was no difference between any of the PDGF treatments, indicating that 10 ng/mL was at or above the concentration that causes maximal contraction of the FPCM. Insulin-like growth factor-1 did not significantly

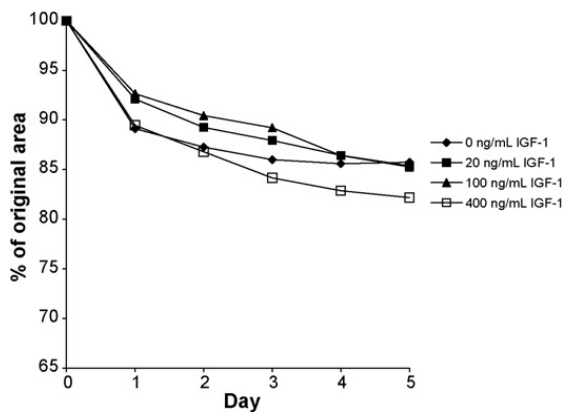


Fig. 2. Effect of insulin-like growth factor-1 on the relative surface area of fibroblast-populated collagen matrix. Least significant difference: 5.065. Split-plot analysis of variance revealed that there was no overall significant difference between the treatments in this experiment, but there was a significant interaction ($P = 0.028$); the control (0 ng/mL) treatment reached a plateau by day 2, but the insulin-like growth factor-1 treatments containing 20 or 100 ng/mL were still contracting on day 5.

affect the average contraction of the FPCM relative to the control over the 5-d period of the experiment (Fig. 2). However, there was a significant interaction ($P = 0.028$) such that the control treatment reached a plateau by day 2, whereas FPCM treated with either 20 or 100 ng/mL IGF continued to contract and were still contracting on day 5. Transforming growth factor $\beta 1$ did not affect FPCM contraction relative to the control at any concentration investigated (Fig. 3).

3.2. Experiment 2: Effect of inhibiting Rho-kinase and p38 on PDGF-stimulated FPCM contraction

Addition of either of the Rho-kinase inhibitors blocked PDGF-stimulated contraction ($P < 0.001$) without affecting basal levels of contraction (Fig. 4). Similarly, addition of either of the p38 inhibitors had a

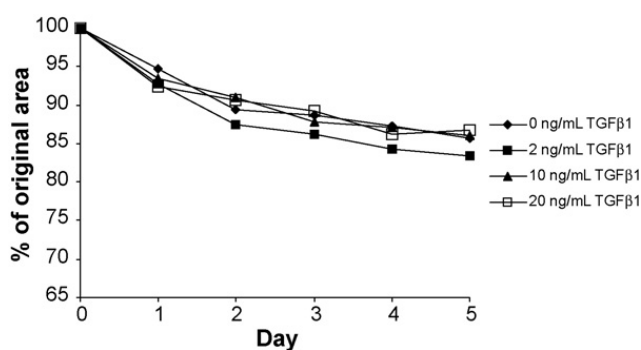


Fig. 3. Effect of transforming growth factor $\beta 1$ on the relative surface area of fibroblast-populated collagen matrix. Least significant difference: 5.980. There was no significant effect of transforming growth factor $\beta 1$ at any concentration tested on the contraction of the gels.

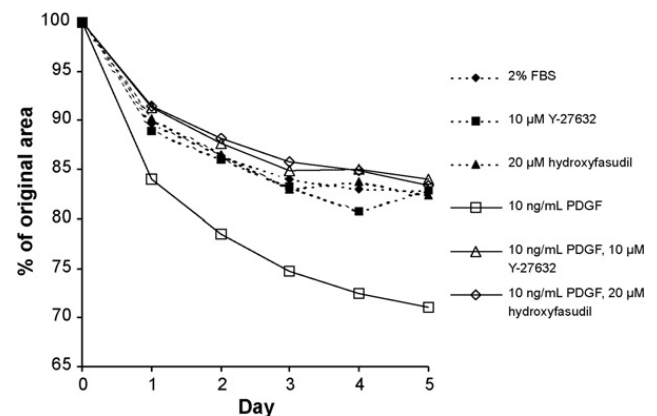


Fig. 4. Effect of the Rho-kinase inhibitors Y-27632 and hydroxyfasudil on platelet-derived growth factor BB (PDGF)-stimulated contraction of fibroblast-populated collagen matrix. Least significant difference: 4.591. Treatment with 10 ng/mL PDGF stimulated a significant increase ($P < 0.001$) in contraction compared to all other treatments, and addition of either of the Rho-kinase inhibitors reduced PDGF-stimulated contraction back to control levels ($P < 0.001$).

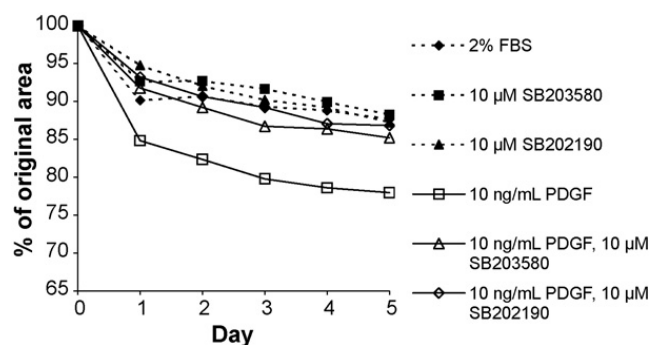


Fig. 5. Effect of the p38 inhibitors SB203580 and SB202190 on platelet-derived growth factor BB (PDGF)-stimulated contraction of fibroblast-populated collagen matrix. Least significant difference: 4.348. Treatment with 10 ng/mL PDGF stimulated a significant increase ($P < 0.001$) in contraction compared to all other treatments, and addition of either of the p38 inhibitors reduced PDGF-stimulated contraction back to control levels ($P < 0.001$).

similar effect in blocking PDGF-stimulated contraction ($P < 0.001$) without affecting basal levels of contraction (Fig. 5). Inclusion of an equivalent amount of DMSO only (in which the p38 inhibitors were dissolved) did not affect contraction (data not shown). These results suggest that both Rho-kinase and p38 are important in the generation of tractional force in FPCM stimulated with PDGF.

4. Discussion

Of the 3 growth factors tested in Experiment 1, PDGF was the only one to significantly stimulate contraction of FPCM, with a maximal effect at 10 ng/mL. These results are in agreement with data published using this

methodology in other species [16,19,20]. Floating matrices contract predominantly because of tractional forces resulting from fibroblast migration [14], and PDGF is a strong chemotactic agent, present in high concentrations early in the healing process [6]. It is therefore likely that PDGF caused contraction of the floating FPCM via its ability to stimulate fibroblast migration and hence increase tractional forces.

IGF-1 did not significantly affect the contraction of the FPCM at any of the concentrations tested. *In vivo*, IGF-1 is normally found bound to one of 6 IGFBP, predominantly IGFBP-3 [21]. Insulin-like growth factor binding proteins have a role in modulating the binding of IGF-1 to its receptor, and they can either strengthen or inhibit the action of IGF-1 [22]. Therefore, delivery of unbound IGF to cells, as was the case here, is likely to have a different effect than if it was presented bound to its binding proteins. Indeed, the addition of free IGF-1 to wounds has been shown to have adverse side effects, including electrolyte imbalance and edema [21]. Lee et al. [17] showed that although IGF-1 on its own at 30 or 100 ng/mL stimulated contraction of FPCM containing human foreskin fibroblasts (6.8% and 7.7% decrease in diameter of gels within 6 h), just 1 ng/mL of IGF-1 combined with 1 ng/mL IGFBP1 led to a decrease in the matrix diameter by 14% within 6 h. Insulin-like growth factor-1 has been shown to stimulate contraction of FPCM containing human heart fibroblasts without IGFBP [23]. Assouline et al. [19] showed that 10 ng/mL IGF-1 in combination with 10 or 100 ng/mL epidermal growth factor (EGF) stimulated contraction of collagen gels containing human keratocytes, though if IGF was combined with either PDGF or fibroblast growth factor, contraction was inhibited. These papers therefore suggest IGF-1 can stimulate contraction but that its effect is dependent on other growth factors and its binding protein.

Transforming growth factor β 1 had no significant effect on the contraction of the FPCM (Fig. 3), despite the fact that it is commonly acknowledged as a key growth factor in the wound contraction process owing to its ability to stimulate myofibroblast differentiation [24]. However, the floating matrices used here do not cause fibroblasts to differentiate because of the low tension within the collagen matrix, and instead, tractional force resulting from fibroblast migration is thought to be the main mechanism stimulating contraction [14]. Andersen et al. [16] and Ellis and Schor [25] demonstrated that TGF β 1 inhibited migration of human keratocytes and dermal fibroblasts, respectively. It is therefore likely that although TGF β 1 did not inhibit fibroblast migration in the present experiment, (as this would have led to a

lower contraction of the gels compared to the control), it did not stimulate contraction either. The concentrations used here have previously been shown to stimulate contraction of FPCM in other species [15,26], and effects on equine cell proliferation have also been shown at these concentrations [27,28]. In the present experiment, results were replicated using multiple batches of TGF β 1 (results not shown), indicating this result was not owing to a denatured batch of TGF β 1.

It has been shown that the equine TGF β 1 amino acid sequence is unique to the horse, with a difference of two amino acids in comparison to some other mammalian species [29]. Although Nixon et al. [29] claim that these amino acid substitutes in the horse are unlikely to lead to major functional differences, if these substitutions occur in the region of the protein that binds to the TGF β receptors, then the human TGF β 1 used in these experiments could have had a reduced binding affinity with equine TGF β receptors, thus leading to the lack of action reported here. A basic local alignment search tool (BLAST) search shows that the structures of TGF β RI and TGF β RII are, respectively, 98% and 100% homologous between the human and equine proteins. If the unique structure of equine TGF β 1 altered its ability to bind to its receptors, then it would be expected that a corresponding structural change would also be observed in the receptor. Therefore, the close homology between the receptors of the two species suggests that equine TGF β receptors should recognize human TGF β 1, although the affinity may still be reduced.

Others have also published papers using TGF β 1 from other species on equine cells [27,28], which also supports the notion that human TGF β 1 will react with equine TGF β receptors. It has also been shown that TGF β 1 does not stimulate contraction in oral mucosal fibroblasts compared to dermal fibroblasts [30], thus the fact that TGF β 1 did not stimulate cells to contract FPCM here may be linked to the use of oral fibroblasts within this experiment. Therefore, the results gained in our study can probably be explained through a combination of the model used and the phenotype of the cells embedded within it.

For each cell signaling pathway investigated, two inhibitors were used, as recommended by Davies et al. [18]. Although the inhibitors chosen here had been well characterized for their specificity, no inhibitor will exclusively inhibit only one protein kinase, but instead they inhibit a range of kinases with varying degrees of specificity [18]. Therefore, if two different inhibitors with different spectra of specificity are used, and both of these inhibitors produce identical results, it gives confidence that the intended pathway being studied is actually

the primary one being inhibited [18]. Both Rho-kinase inhibitors Y-27632 and hydroxyfasudil reduced the contraction of PDGF-stimulated FPCM to levels observed in the controls (Fig. 4), suggesting that Rho-kinase plays a vital role in mediating the effect of PDGF. This is the first time that the effect of Rho-kinase inhibitors on equine cells has been investigated. Nevertheless, these results are in agreement with those for PDGF-stimulated FPCM treated with Rho-kinase inhibitors in other species [7,8].

Rho-kinase is an essential enzyme in the formation and contraction of actin stress fibers that are important in the generation of tractional force [31]. In addition to stress fibers, Rho-kinase activity is intimately linked to the expression of focal adhesions, and inactivation of Rho-kinase leads to rapid loss of integrin clusters on the cell surface [32]. Crucially, however, when fibroblasts are embedded into floating collagen gels, the low tension leads to an automatic loss of stress fibers and focal adhesions [33], which is probably a result of natural inhibition of Rho-kinase activity owing to decreased cytoskeletal tension [34]. This finding suggests that inhibiting Rho-kinase does not disrupt stress fiber formation in floating gels because there are none to disrupt, but that instead Rho-kinase must have an alternative effect.

Grinnell [35] suggests that the requirement for Rho-kinase in floating matrices might be a reflection of the requirement of the cell to maintain cytoskeletal integrity. Rho-kinase has been implicated in regulation of microtubule dynamics [36]. Contraction of floating FPCM containing a low to moderate cell density, as was the case in the experiments in this study, is dependent on microtubules [37]. Inhibitor Y-27632 has been shown to alter the organization of microtubules in mice [38], and this change in morphology may be enough to alter the ability of the cell to transmit force to the matrix. More research investigating the effect of Rho-kinase inhibitors on microtubule dynamics in equine fibroblasts embedded in floating FPCM is needed to elucidate the mechanism of inhibition.

Inhibitors SB203580 and SB202190 also blocked the contraction of PDGF-stimulated FPCM back to control levels (Fig. 5), suggesting that p38 also plays a vital role in mediating PDGF-stimulated contraction. Platelet-derived growth factor is a strong stimulator of mitogen-activated protein kinase (MAPK) pathways, including p38 [7]. This is the first time that inhibition of p38 has been studied using equine cells. Both Hirano et al. [9] and Li et al. [7] reported significant inhibition of contraction of FPCM by SB203580 and SB202190 on rat fibroblasts and bovine aortic smooth muscle cells, respectively. However, in both papers, the contraction was not reduced to the levels seen in the control FPCM.

This finding is possibly a result of differences in the treatments, model, and/or species; Hirano et al. [9] used 2.5% FBS or thrombin and FPCM that had been attached for 3 d, and Li et al. [7] used 10% FBS and a cell concentration over 10 times higher than that used here. It can therefore be concluded that stimulation of p38 by PDGF is an important signaling cascade responsible for the generation of tractional forces in FPCM.

5. Conclusions

The results presented here show that in floating FPCM containing equine oral fibroblasts, PDGF is a potent stimulator of contraction, whereas neither IGF-1 nor TGF β 1 stimulated contraction in this model. Platelet-derived growth factor appears to stimulate this contraction via both the Rho-kinase and p38 signaling pathways. This experiment has established some of the growth factors and signaling pathways that potentially control the initiation of wound contraction via tractional force in a model containing oral fibroblasts, which thus represents a wound that would heal scarlessly. We intend to continue this research using fibroblasts obtained from the limbs of horses, because of the poor healing normally seen on the legs of horses relative to the buccal cavity. Differences between the two models could provide insight into why limb wounds heal so poorly, and whether manipulation of both the growth factor profile and the Rho-kinase and p38 signaling pathways may provide a method of promoting wound contraction in vivo.

Acknowledgments

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